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# Phosphodiesterase 5/protein kinase G signal governs stemness of prostate cancer stem cells through Hippo pathway

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ABSTRACT

Cancer stem cells (CSC) are critical for initiation, metastasis, and relapse of cancers, however, the underlying mechanism governing stemness of CSC remains unknown. Herein, we have investigated the roles of phosphodiesterase 5 (PDE5) in stemness of prostate cancer cells. Both PDE5 and WW domain-containing transcription regulator protein-1 (TAZ), a core effector of Hippo pathway, are highly expressed in the PC3-derived cancer stem cells (PCSC). Either TAZ knockdown or inhibition of PDE5 activity attenuated colony formation, altered expression patterns of stem cell markers, and enhanced cisplatin cytotoxicity, resulting in attenuation of stemness in PCSC. In addition, inhibition of PDE5 activity by its specific inhibitors activates cGMP-dependent protein kinase G (PKG), which in turn induces MST/LATS kinases, resulting in cytosolic degradation of TAZ and activation of Hippo pathway. Accordingly, knockdown of TAZ almost completely abolished PDE5 inhibitor-induced attenuation in stemness in cultured PCSC, whereas knockdown of TAZ not only abolished PDE5 inhibitor-induced attenuation in stemness but also facilitated PDE5 inhibitor-induced trans-differentiation in PCSC xenografts. Together, the present study has uncovered that PDE/cGMP/PKG signal targets to Hippo/TAZ pathway in maintaining stemness of PCSC, and suggested that PDE5 inhibitors in combination with chemotherapeutic agents could effectively prevent initiation, metastasis, and relapse of prostate cancer.



## Introduction

Prostate cancer is a frequently diagnosed and relapsed male malignancy. Recently, a tiny population of prostate cancer cells characterized by slow growth, self-renewing and asymmetric division, had been isolated and defined as prostatic cancer stem cells (PCSC) [1,2]. Like other cancer stem cells, PCSC are the emerging interpretation for prostate cancer initiation, metastasis, relapse and chemoresistance [3–5], however, the molecular mechanisms governing the stemness and differentiation of PCSC remain elusive.

Hippo signaling pathway has conserved roles in metazoans ranging from Drosophila to humans [6,7]. In mammals, cell–cell junctions and apicobasal polarity are involved in upstream activation of Hippo cascade, and the core to Hippo pathway is a kinase cascade, wherein Ste20-like kinases, Mst1/2, phosphorylate and activate the nuclear dbf2-related family kinases, Lats1/2. Lats1/2 kinases in turn phosphorylate two major downstream effectors, Yes-associated protein (YAP) and transcriptional co-activator with PDZ-binding motif (TAZ), resulting in their ubiquitination and proteolysis [8,9]. On the country, silence of Hippo signaling enhances nuclear location of YAP and TAZ, which subsequently bind to Sd homologs TEAD1/4 and other transcription factors to promote transcription of target genes, such as connective tissue growth factor (ctgf) and cysteine-rich angiogenic inducer 61 (cyr61) [6,7,10]. The Hippo pathway plays crucial roles in not only contact inhibition, organ size control and stem cell maintenance but also cancer stem cell maintenance, and Hippo

*Abbreviations:* 5'-GMP, guanosine-5'-monophosphate; cGMP, cyclic guanosine monophosphate; CSCs, cancer stem cells; ED, erectile dysfunction; eNOS, endothelial NOS; GC, guanylyl cyclase; iNOS, inducible NOS; LATS, nuclear dbf2-related family kinases or large tumor suppressor kinase; MST, mammalian ste20-like protein kinase; NO, nitric oxide; NOS, nitric oxide synthase; PCSC, prostate cancer stem cells; PDE5, phosphodiesterase 5; PKG, cGMP-dependent protein kinase; PSA, prostate specific antigen; sGC, soluble guanylyl cyclases; TAZ, WW domain-containing transcription regulator protein 1; TEADs, TEA domain family members; YAP, Yes associated protein.

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pathway disruption is closely associated with poor outcome in a variety of malignancies [7,11]. TAZ is elevated in high-grade breast cancer and contributes to breast cancer stem cell self-renewal [12]. YAP but not TAZ up-regulates CD90, a hepatocellular carcinoma-specific cancer stem cell marker, resulting in tumorigenesis and chemoresistance in hepatocellular carcinoma [13]. In addition, YAP is activated by ETS-related gene (ERG), and contributes to development of age-related prostate tumors [14], whereas aberrantly overexpressed YAP sufficiently transforms LNCaP prostate cancer cells to an androgen-insensitive state responsible for castration resistance [15]. However, whether Hippo tumor suppressor pathway is correlated with stemness and differentiation of PCSC remains elusive.

Cyclic guanine monophosphate (cGMP) activated by soluble guanylyl cyclases and degraded by cyclic nucleotide phosphodiesterases (PDEs) mediates biological signaling by nitric oxide (NO). cGMP binds and activates cGMP-dependent protein kinases (PKGs), which phosphorylate serine and threonine residues on many cellular proteins, resulting in changes in their activity, subcellular localization or regulatory features [16,17]. Current therapies targeting the nitric oxide (NO)-signaling pathway include nitrovasodilators, such as nitroglycerin and sodium nitroprusside, and PDE5 inhibitors, such as sildenafil, vardenafil, and tadalafil for treatment of a number of vascular diseases including angina pectoris, erectile dysfunction, and pulmonary hypertension [18,19]. Recently, several studies have hinted at the possible effect of NO/cGMP on stem cell differentiation [20,21]. NO synthetase (NOS) inhibitors increase the number of bone marrow derived hematopoietic stem cells, whereas NO signaling promotes differentiation of embryonic stem cells into myocardial cells [22]. Moreover, PDE5 inhibition improves adipose-derived stem cells survival, and the combination of PDE5 inhibition and adipose-derived stem cells benefits to the rehabilitation of myocardial infarction [23]. Finally, because elevated PDE5 expression has been reported in multiple human carcinomas and in many carcinoma cell lines [24,25] and its elevation occurs with increasing tumor grade and stage, PDE5 has been suggested to be involved in tumor initiation and progression [26]. Accordingly, PDE5 inhibitors are believed to be potent anticancer drugs with a novel mechanism of actions including enhancing the apoptosis, anti-proliferation, efficacy of chemotherapy and attenuating chemoresistance and multi-drug resistance [27,28]. However, whether PDE5 and its inhibitors contribute to the stemness and differentiation of PCSC remains largely elusive.

In the present study, we have investigated the underlying mechanisms by which PDE5/cGMP/PKG signal governs stemness and differentiation of PCSC. We have found that inhibition of PDE5 enhances the differentiation and attenuates the stemness of PCSC through activation of Hippo/TAZ signaling pathway.

## Materials and methods

### *Cell lines and culture*

Prostatic carcinoma cell lines, including PC3 and DU145 cells were purchased from ATCC (Manassas, VA). PC3 cells were maintained in Dulbecco's Modified Eagle Medium: Nutrient Mixture F-12 (DMEM/F-12) containing 1 × GlutaMAX™, 7% fetal bovine serum (Life Technologies, Inc., Carlsbad, CA), 100 U/ml penicillin, and 100 μg/ml streptomycin. DU145 cells were maintained in RPMI-1640, 7% fetal bovine serum, 100 U/ml penicillin, and 100 μg/ml streptomycin. All cell lines were incubated at 37 °C with 5% CO2.

### *Isolation of PC3-derived PCSC by limiting dilution*

The PC3 derived prostate cancer stem cells were isolated and purified as previously described [1,2]. In belief, PC3 cells were harvested and washed with FBS-free DMEM/F-12 medium twice, then re-suspended in DMEM/F-12 medium containing 5% FBS and 1 × GlutaMAX™ (Invitrogen) to generate a single-cell suspension with a density of 10 cells/ml. 100 μl/well single-cell suspension was plated into 96-well plates. After 24 h, wells containing only a single cell were marked and further maintained in DMEM/F-12 medium containing 5% FBS and 1 × GlutaMAX™. After 3–4 weeks, cells grew to confluence and the culture medium was replaced with PC3 cell growth medium, then, each clone was expanded and subjected to identification of stem cell-like properties.

### *Western blot assays*

Cells were seeded to 6-well plate at 70–80% confluence, and cultured for 48 h, then treated with or without indicated concentrations of vardenafil HCl trihydrate (Selleckchem, Houston, TX) for further indicated time for western blot. Protein extracts from cells or tumor samples were prepared in whole cell lysis buffer (50 mM HEPES, 150 mM NaCl, 1 mM EGTA, 10 mM sodium pyrophosphate, 1.5 mM $MgCl_2$, 100 mM sodium fluoride, 10% glycerol, and 1% TritonX-100) containing an inhibitor mixture (1 mM phenylmethysulfonyl fluoride, 10 μg/ml aprotinin, and 1 mM sodium orthovamadate). Protein concentrations were determined using a standard Bradford assay, and 50 μg of total protein was subjected to SDS-PAGE followed by a transfer onto 0.45 μm PVDF membranes (Millipore, Bedford, MA). Membranes were incubated overnight at 4 °C with primary antibodies. Antibodies against p-TAZ, TAZ, YAP, PKG2, Nanog, Lamin B, Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin were from Santa Cruz Biotechnology (Santa Cruz, CA), antibodies against p-MST, p-LATS, p-YAP, MST1, and LATS1 were from Cell Signaling (Danvers, MA), antibodies against PDE5a and PKG1 were obtained from Abcam (Cambridge, UK), antibodies against Sox2 and CTGF were from BBI Life Sciences Corporation (Shanghai, China), and antibodies against myc-tag, HA-tag and flag-tag were from TransGen Biotech (Beijing, China). The IRDye 680 and 800 second antibodies were purchased from LI-COR Bioscience (Lincoln, Nebraska). The immunoreactive signals were visualized with Odyssey Infrared Imaging System (LI-COR, Lincoln, NE), and GAPDH or β-actin was used as internal standards. ImageJ software from National Institutes of Health (http://rsb.info.nih.gov/ij/download.html) was used to quantify the immunoreactive bands, and the mean intensity of the first band was set to 1.

### *RNA isolation and quantitative RT-PCR*

Cells were seeded using a 6-well plate and at 70–80% confluence, cultured for 48 h, and then treated with or without indicated concentrations of vardenafil for a further 6 h for RT-qPCR. Total RNA was isolated by using Trizol reagent (Takara Biotechnology Co., Ltd., Dalian, China) according to the manufacturer's instructions. 1 μg total RNA in a volume of 20 μl was reversely transcribed by using SuperScript III reagent (Life Technologies). After termination of cDNA synthesis, each reaction mixture was diluted with 80 μl Tris-EDTA buffer. Messenger RNA levels of target genes were determined by quantitative RT-PCR as previously described [29]. The relative amounts of the mRNA levels of the target genes were normalized to the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and β-actin levels, respectively, and the relative difference in mRNA levels was calculated by $2^{-\Delta\Delta Ct}$ method.

### *Cell counting kit-8 assays*

Cells were seeded into 96-well plates at 1000 cells/well, and cultured for 8 days. Cellular vitality was measured every two days by cell counting kit-8 (Beyotime, Shanghai, China) as per manufacturer's instructions. In chemoresistance experiments, cells were seeded into 96-well plates at 5000 cells/well, after culture for 24 hours, cells were treated with indicated concentrations of Cisplatin (Sigma, St. Louis, MO) for a further 48 hours, and then cellular vitality was measured by cell counting kit-8. The cytotoxicity (%) = [1 – (OD from tested cells)/OD from control cells]*100%.

### *Mammosphere formation assays*

Mammosphere formation assays were performed as previously described [2]. In belief, cells were harvested and washed with FBS-free DMEM/F-12 medium twice, then re-suspended in FBS-free DMEM/F-12 medium containing 1 × B27 (Invitrogen), 20 ng/ml human bFGF (PeproTech, Rocky Hill, NJ), 20 ng/ml human EGF (PeproTech), and 3 μg/ml insulin (Sigma, St. Louis, MO). After that, single cells were seeded into 6-well Ultra–Low Attachment plates (Corning, NY) at a density of either $5 \times 10^4$ or $1 \times 10^5$ cells/well and half volume of medium was carefully changed every 2 days. After two weeks, spheres were captured by Olympus fluorescence microscope.

### *Clone formation assays*

Clone formation assays were performed as previously described [2]. In belief, cells were harvested and washed twice, then re-suspended in 5% FBS-free DMEM/F-12 medium containing 1 × GlutaMAX™; single cells were plated in 6-well plates at a density of 1000. The medium containing vehicle or vardenafil was carefully changed every 2 days for two weeks, then cells were washed twice and stained with crystal violet. Clones of each well were captured and subsequently analyzed by ClonCounter software [30].

### *Fluorescence-activated cell sorting*

Cells were re-suspended at a density of $1 \times 10^6$ cells/ml, and then intracellular NO were labeled by 5 μM 3-Amino,4-aminomethyl-2',7'-difluorescein, diacetate